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PATHWAYS OF GLUCOSE CATABOLISM IN *CALDARIOMYCES FUMAGO* (ILL.)

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SUMMARY

Caldariomyces fumago is unusual in that it utilizes parts of a number of pathways in glucose catabolism to pyruvate. The initial oxidation of glucose is through the "notatin" type of oxidation to 2-ketogluconate. This then probably proceeds to 6-phosphogluconate via the adaptive enzymes 2-ketogluconic kinase and 2-keto-6-phosphogluconic-reductase. The 6-phosphogluconate proceeds to form pyruvate via the rest of the Entner–Doudoroff pathway, hexose monophosphate pathway and Embden–Meyerhof pathway. The terminal oxidation of pyruvate occurs by the usual tricarboxylic cycle. Thus, *Caldariomyces fumago* does not possess all the enzymes of any of the three normal pathways of glucose catabolism, the Entner–Doudoroff pathway, hexose monophosphate pathway or Embden–Meyerhof pathway, but utilizes part of the enzyme systems in the complete utilization of glucose.

INTRODUCTION

In the ever expanding field of cellular biology an organism with an unusual combination of the known pathways contributes to our knowledge on the role of individual pathways in the total cell metabolism. Evidence is presented here for the existence of such an unusual combination of known pathways in the fungus *Caldariomyces fumago*. As far as could be ascertained from the literature, evidence is presented here for the first time on the fate of 2-ketogluconic acid in the glucose metabolism of fungi. *C. fumago* belongs to the order Moniliales of the Imperfect Fungi; it occurs commonly as sooty mold of greenhouse plants in Northern Europe[1]. Studies on the metabolism of fungi are basically important in plant pathology for they could help in better understanding host–parasite relationships.

METHODS

The stock culture of *C. fumago* (Ill.) was obtained from Dr. H. RAISTRICK, Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London. The fungus was routinely subcultured on oatmeal agar slants. Inocula for liquid cultures were prepared by asceptically transferring spores and some mycelium

Abbreviations: E–M pathway, Embden–Meyerhof pathway; H.M.P. pathway, hexose monophosphate pathway; E–D pathway, Entner–Doudoroff pathway; C.F.P., cell-free preparation.

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with an inoculating needle to 100 ml of modified Czapeck-Dox medium* in a 500-ml Erlenmeyer flask. The culture was grown at 26° on a reciprocal shaker at 100 strokes/min. After the culture had grown for 3–4 days, the contents were blended for 40 sec in a sterilized Waring blendor and fresh medium (100 ml) was inoculated with 2.5 ml of the blended culture. The organism was maintained in shake flask liquid culture. Mycelia for the experiments were prepared as follows: the mycelium from the shake cultures was centrifuged, the supernatant liquid discarded, and the mycelium washed twice with water and once with phosphate buffer solution, the pH of which was adjusted to that of the culture fluid with hydrochloric acid or sodium hydroxide solution. The washed mycelium was resuspended in 100 ml of the buffer and blended for 40 sec to procure a uniform, mycelial suspension that could be pipetted. The composition of the phosphate buffer used in all experiments, unless otherwise mentioned, was as follows: KH_2PO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and distilled water to make 1000 ml.

All manometric techniques used were as described by UMBREIT *et al.*[2]. Phosphate determinations were by the method of FISKE AND SUBBAROW[3]. Pyruvate was analyzed by the method of FRIEDMAN AND HAUGEN[4]. Glucose was determined by the manometric method of KAPLAN[5]. α-Ketoacids were determined by the ceric sulfate method of KREBS AND JOHNSON[6]. Barium salts of substrates studied were converted to either the potassium or magnesium salts before use.

Following is the method used for the confirmation of pathways of glucose breakdown using [1-^{14}C]glucose. A conventional Warburg apparatus with double side-arm flasks was used throughout the experiment. The center-well of the flask contained 0.3 ml of 20% KOH, but no paper. The main body of the flask contained 1 ml blended hyphae from a 54.5-h old culture with a final pH of 6.0, sodium arsenite to give a final concentration of $3 \cdot 10^{-3}$ M, and phosphate buffer to make a final fluid volume of 3.2 ml. One side-arm contained 28 μmoles of either "cold" glucose or [1-^{14}C]glucose with a specific activity of 1.63 μC/μmole, the other side-arm contained 0.2 ml of 30% trichloroacetic acid. The water-bath was maintained at 29.6°. After equilibration, glucose was tipped in.

At the end of 1, 3, 5, and 6 h, the trichloroacetic acid was tipped into the various sets of flasks to stop the enzymic reaction. After the reaction has been stopped in the 6-h flasks, all flasks were allowed to shake an additional 60 min to ensure that all the carbon dioxide was absorbed by the KOH. The flasks were then removed and 20-μl samples in duplicate of KOH from the center-well were pipetted into scintillation bottles from all the flasks that contained [1-^{14}C]glucose. The scintillation bottles were kept at 4°. Then the suspension in the main body of the Warburg vessel was carefully removed without disturbing the KOH. In order to remove all the material, the flask was rinsed twice with 2 ml of distilled water. The combined suspensions were centrifuged, the supernatant liquid concentrated under vacuum, the concentrate streaked on a Whatman No. 1 chromatographic paper, and the chromatograms developed in a water-saturated butanol–formic acid (95:5) system. After air-drying, the area of chromatogram containing the pyruvic acid was cut out, and the pyruvic acid eluted with the same solvent system. Chromatography was necessary to separate the 2-keto-

* D-Glucose, 10.0 g; $NaNO_3$, 2.0 g; KH_2PO_4, 1.0 g; KCl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; distilled water to make 1000 ml. Final pH of medium 4.5. Medium was sterilized by autoclaving at 15 lb pressure for 15 min.

gluconate from pyruvate, because ceric sulfate would decarboxylate 2-ketogluconate just as well as pyruvate. The eluate was evaporated to dryness and then redissolved in 2.0 ml of distilled water. All of this 2.0 ml was transferred to a conventional Warburg flask which contained 0.3 ml of 20% KOH in the center-well, but no paper. Into the main body of the flask was added 0.5 ml of 1:1 aqueous sulfuric acid. The side-arms contained 0.6 ml of a saturated solution of ceric sulfate (recrystallized) in 2 N sulfuric acid. After 15 min equilibration, the ceric sulfate solution was tipped in. The water-bath temperature was 29.6°. After 2 h, the flasks were removed and 20-μl duplicate samples of KOH were transferred from the center-well into scintillation bottles. Two 20-μl samples were also taken from the [1-^{14}C] glucose solution used in the experiment to determine its specific activity. To each scintillation bottle 7 ml of absolute alcohol, 8 ml of diphenyloxazole in toluene, and 0.5 ml of distilled water were added and the radioactivity in each bottle was counted in a liquid scintillation counter. The first set of KOH samples that contained the carbon dioxide from the oxidation of C-1 of glucose by the hexose monophosphate pathway are called Set 1 and the second, containing the carbon dioxide from decarboxylation of pyruvate are called Set 2. The results of this experiment are given in Table VI.

Dry weights were determined by drying samples in an oven at 80° for 48 h and weighing. Protein determinations were made by the micro-Kjeldahl method, and the pH measurements were made with a Beckman pH meter.

RESULTS

Aerobic and anaerobic oxidation of glucose

The aerobic and anaerobic oxidation of glucose by the washed cells and cell-free preparation were studied by the standard manometric techniques[2]. It is evident from Table I that the whole cells and cell-free preparation exhibit no anaerobic oxidation of glucose. The lack of CO_2 production even in the presence of the bicarbonate buffer indicates that no acids were formed from glucose. It is important to note that under

TABLE I

AEROBIC AND ANAEROBIC OXIDATION OF GLUCOSE BY WHOLE CELLS AND CELL-FREE PREPARATIONS

The complete system contained in a final volume of 3 ml, 0.01 M $MgSO_4 \cdot 7H_2O$, 0.1 ml; 0.01 M glucose, 0.2 ml; washed cell suspension in buffer from a 4-day culture with a pH of 7.0, 0.5 ml; or cell-free preparation, 0.5 ml; 0.02 M potassium phosphate buffer (pH 7.0) or 0.01 M sodium bicarbonate buffer pH of 7.0 to make up to the final volume. The values for endogenous respiration have been subtracted in each case. The cell-free preparation (C.F.P.) was made as follows: 18 g washed cells were ground with 20 g of washed 3F grade carborundum for 4 min. To this, 20 ml of 0.02 M phosphate buffer (pH 7.0) were added and the suspension was mixed well. The homogenate was centrifuged at 1500 × *g* for 20 min. The supernatant fraction, with 7.0 mg protein/ml, was used as the cell-free preparation. All the material used was precooled to 4° and the entire operation was done at 4°.

Expt.	*Aerobic or anaerobic (gas phase)*	*Buffer*	*Addition*	*μl O_2 uptake/h*	*μl CO_2 liberated*
1	Air	KPO_4	Whole cells	230.5	—
2	N_2	KPO_4	Whole cells	—	3.2
3	N_2	$NaHCO_3$	Whole cells	—	12.2
4	Air	KPO_4	C.F.P.	66.5	—
5	N_2	KPO_4	C.F.P.	—	0
6	N_2	$NaHCO_3$	C.F.P.	—	0

aerobic conditions the cell-free preparation consumed only 66.5 μl of O_2/2 μmoles of glucose.

Effect of inhibitors on glucose oxidation

Table II summarizes the effect of inhibitors on the O_2 uptake by whole cells and cell-free preparations with glucose as substrate. Though all the inhibitors except fluoride and malonate partially inhibited O_2 uptake of whole cells, none of them had any discernible effect on the respiration of the cell-free preparation. The cell-free preparation utilized only about 1.5 μmoles O_2/μmole of glucose. Whenever whole cell respiration was inhibited the O_2 uptake was close to that of the cell-free preparation. These observations indicated that glucose went through some initial oxidation that was insensitive to cyanide and azide as well as to the other inhibitors. It was also evident that this cyanide-insensitive respiration occurred early in the glucose oxidation, because (a) whole cell respiration was partially cyanide-sensitive (b) the C.F.P. which could only partially oxidize glucose was not inhibited by cyanide, and (c) other inhibitors of glucose metabolism, when they had an effect on the whole cells, did not stop this cyanide-insensitive respiration.

TABLE II

EFFECT OF INHIBITORS ON GLUCOSE OXIDATION BY WHOLE CELLS AND CELL-FREE PREPARATION

Assay system was the same as in Table I. To each flask 4 μmoles glucose was added, and the experiment was run at 29.6° for 60 min. In all cases except those marked with the asterisk, the O_2 uptake came to a standstill. Method of cell-free preparation same as in Table I.

Inhibitors and their final concentration		*μl O_2 uptake by whole cells*	*μl O_2 uptake by the cell free preparation (3.5 mg protein)*
No inhibitor		210*	121
Potassium cyanide	0.001 M	124	116
Sodium azide	0.001 M	130	118
Sodium fluoride	0.02 M	200*	120
Iodoacetic acid	0.001 M	104	110
Sodium arsenite	0.001 M	103	112
Malonate	0.01 M	200*	120

The identification of the products formed from glucose oxidation by C.F.P.

The cyanide, azide-insensitive aerobic oxidation of glucose resembled the glucose oxidase of Muller from *Penicillium glaucum* and later described by KEILIN AND HARTREE in *P. notatum*[7]. Gluconic acid and 2-ketogluconate are reported to be the products of glucose catabolism in *P. notatum*. In order to determine the products of glucose oxidation by cell-free preparations of *C. fumago*, an experiment was set up as follows: C.F.P., 1.0 ml, glucose 10 μmoles, and phosphate buffer 2.0 ml were placed in a 125-ml Erlenmeyer flask and incubated at 30°. Duplicate flasks were taken off at the end of 30 and 60 min. In the control flasks the C.F.P. was replaced with phosphate buffer. The solutions were concentrated *in vacuo* and chromatographed (descending) on Whatman No. 1 paper in two different solvent systems: (a) ethanol–methanol–water (9:9:2); (b) ethanol–methanol–water–formic acid (15:15:9:2). The chromatograms were removed from the solvents, air-dried, sprayed with 0.1 N $AgNO_3$ solution in 5 N NH_4OH, and then exposed to H_2S. The R_F of the spots were compared

with the standards glucose, gluconic acid and 2-ketogluconic acid. In the 30-min samples, glucose, gluconate and 2-ketogluconate spots were present. In the 60-min sample, 2-ketogluconate, traces of gluconate but no glucose could be detected.

Effect of catalase on the glucose oxidation by whole cells

Since it was repeatedly observed that glucose was oxidized only to 2-ketogluconic acid by cells from liquid cultures with pH of 5 or lower, it was considered possible that this block might be due to the inhibition of the enzymes that catabolized 2-ketogluconate further, by the hydrogen peroxide formed by the glucose oxidase while oxidizing glucose to gluconic acid. In a cell, under normal growing conditions, enough catalase is probably produced to break down this H_2O_2. In cultures with pH of 5 or lower, catalase might be present only in insufficient amounts for the detoxification. Experiments were set up to check this hypothesis (Table III). The marked stimulatory effect of added catalase on the oxidation of glucose can be seen from Table III. The experiment was repeated several times with similar results.

The data indicated that all the glucose catabolized went through the glucose $\longrightarrow$ gluconate $\longrightarrow$ 2-ketogluconate pathway and that H_2O_2 was produced in the first step oxidation of glucose.

TABLE III

EFFECT OF CATALASE ON GLUCOSE OXIDATION BY WHOLE CELLS

To 10 ml whole cell suspension* in phosphate buffer (pH 6.8) 1 mg of catalase was added and mixed well. The ability of these cells to oxidize glucose was tested in a Warburg apparatus. The system contained in addition to buffer, 2 μmoles of glucose and 10 μmoles each of ferrous ammonium sulphate and cysteine.

	Cells without catalase	*Cells with catalase*
μl O_2 uptake in 5 h	68	228
μmoles O_2 uptake in 5 h	3	10.3
μmoles O_2 uptake/mole glucose	—	5.15

* The cells were harvested from a culture with pH 4.0.

Utilization of various intermediates

Several six-carbon intermediates of the glucose metabolism were tried for utilization. Table IV summarizes the results. There is practically no utilization of glucose 1-

TABLE IV

UTILIZATION OF SIX-CARBON INTERMEDIATES OF GLUCOSE METABOLISM

The assay system is the same as in Table I (aerobic experiments). Experiments were stopped when the rate of O_2 uptake in flasks with the substrates were same as the endogenous rate of O_2 uptake.

Substrate		*Total μmoles of O_2 uptake*	*μmoles of O_2 consumed per μmole of the substrate*	*μmoles of O_2 uptake in the presence of $1 \cdot 10^{-3}$ M KCN*
Glucose	4 μmoles	6.3	1.57	6.1
Glucose 1-*P*	2 μmoles	1.0	0.5	0.8
Glucose 6-*P*	2 μmoles	0	0	0
Fructose-1, 6-P_2	2 μmoles	0	0	0
Gluconic acid-δ-lactone	4 μmoles	3.0	0.75	3.0
6-Phosphogluconate	6 μmoles	48.0	8.0	0
2-Ketogluconate	4 μmoles	25.0	6.25	0
Glucuronic acid	2 μmoles	0	0	0

phosphate, glucose 6-phosphate, fructose 1,6-diphosphate or glucuronic acid by whole cells. Glucose and gluconic are only partially utilized (probably due to low catalase levels and low levels of induced enzymes). Only two of the substrates tried, 2-ketogluconate and especially 6-phosphogluconate were utilized well. Furthermore, in the utilization of 2-ketogluconate, a lag was always evident; however, this lag was absent when 6-phosphogluconate was the substrate. This lag period disappears when the cells are first incubated with 2-ketogluconate and then washed (Fig. 1). From these data

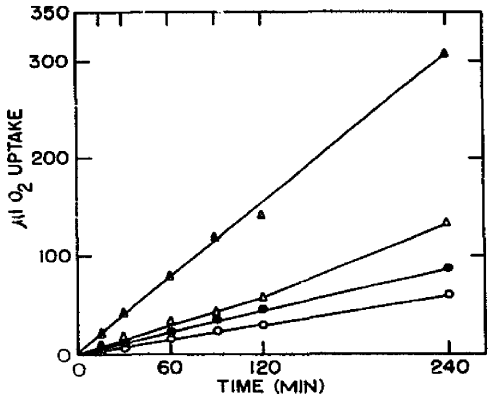

Fig. 1. Effect of preincubation of the mycelium in 2-ketogluconate on the oxidation of the substrate. The mycelium from a culture with a pH of 7.4 was washed twice in distilled water and once in phosphate buffer (pH 7.4) and resuspended in 50 ml of the same buffer in a flask containing 100 μmoles of 2-ketogluconate. The flask was set on a reciprocal shaker for 5 h. After this preincubation the mycelium was washed two times in distilled water and once in the buffer and blended in a Waring blendor for 40 sec. These blended cells were used for respiratory studies in Warburg flasks with 4 μmoles of 2-ketogluconate as substrate. The nonpreincubated cells served as control. ○—○, endogenous; ●—●, endogenous, (preincubated); △—△, with 4 μmoles of 2-ketogluconate; ▲—▲, with 2-ketogluconate (preincubated).

one could infer that the 2-ketogluconate formed from glucose might be converted to 6-phosphogluconate by adaptive enzymes. Such enzymes, which are found in some bacterial systems[8–12], would be, first a kinase that phosphorylates 2-ketogluconate to 2-ketophosphogluconate and then a reductase, that reduces the 2-ketophosphogluconate to 6-phosphogluconate. The conclusion that gluconate, 2-ketogluconate and 6-phosphogluconate are situated in the same direct pathway of glucose utilization by *C. fumago* is further supported by the fact that similar amounts of pyruvate were produced by *C. fumago* from comparable amounts of all these substrates (Table V).

The ratio of 1:1.7 of substrate utilized to pyruvate formed in the presence of $3 \cdot 10^{-3}$ M sodium arsenite indicates that an E–D type pathway might be the major operative pathway of carbohydrate metabolism in *C. fumago*. However, if the E–D

TABLE V

PYRUVATE FORMATION FROM GLUCOSE, 2-KETOGLUCONATE AND 6-PHOSPHOGLUCONATE BY WHOLE CELLS

The complete assay system in a final volume of 3 ml contained washed whole cell suspension in buffer at pH 6.8, 1.0 ml; ferrous ammonium sulfate, 10 μmoles; glutathione, 10 μmoles; substrate, 4 μmoles, and phosphate buffer, pH 6.8. One of the duplicate sets for each substrate contained sodium arsenite to give a final concentration of $3 \cdot 10^{-3}$ M. At the end of the experimental period the enzymic process was stopped in each flask by the addition of 3 ml of 10% trichloroacetic acid. The amount of pyruvate in each flask was determined by the method of FRIEDEMANN AND HAUGEN.

Addition	*μmoles substrate utilized*	*μmoles pyruvate formed*	*substrate: pyruvate*
Glucose	1.68	2.75	1:1.65
2-Ketogluconate	1.94	3.3	1:1.71
6-Phosphogluconate	2.05	3.38	1:1.65

pathway were the sole pathway, then 2 moles of pyruvate must be produced for every mole of substrate utilized. If one assumes that both E–D pathway and H.M.P. pathway operate simultaneously, then calculations from data in Table V indicate that about 65% of the substrates were metabolized by an E–D type pathway and about 35% via a H.M.P. type pathway; 6-phosphogluconate would be the common intermediate for both these pathways. Subsequent experiments with [^{14}C]glucose confirmed the assumption that both these pathways are operative in *C. fumago*.

Experiments with [1-^{14}C]glucose

The number one carbon of glucose, when oxidized via the E–D pathway, ultimately becomes the carboxyl carbon of the pyruvic acid. The E–D pathway is the only pathway by which this process is known to occur in the direct oxidation of glucose. In the H.M.P. pathway the number one carbon of glucose is oxidized to CO_2 before the pyruvic acid is formed. Thus, with the use of [1-^{14}C]glucose, the extent of participation of these two pathways could be determined. The results of such experiments (Table VI) indicate that both E–D and H.M.P. pathways are operative in *C. fumago*.

TABLE VI

OXIDATION OF [1-^{14}C]GLUCOSE BY WHOLE CELLS

Total assay system contained cell suspension, 1.0 ml; where present, sodium arsenite to give a final concentration of $3 \cdot 10^{-3}$ M; glucose, 28 μmoles glucose or [1-^{14}C]glucose with a specific activity of 1.63 μC/μmole. Reaction was stopped by tipping in 0.2 ml of 30% trichloroacetic acid from the side arm. The complete method used is described under METHODS.

Time (h)	*Glucose added (μmoles)*	*Glucose utilized** (μmoles)*	*Desintegrations*/min in set 1 KOH****	*Desintegration*/min in set 2 KOH****	*Percent of glucose utilized was catabolized via*	
					E–D pathway	*H.M.P. pathway*
1	28	1.21	6 000	37 800	86.2	13.8
3	28	7.6	108 000	167 000	61	39
5	28	7.6	97 000	175 000	65	35
6	28	9.2	122 000	228 000	64	36

* Disintegrations/min in 0.3 ml KOH in the center-well.
** These figures were calculated both from the O_2 uptake and from the radioactivities of glucose added and CO_2 absorbed in KOH. Good agreement was found between both these calculations.
*** Set 1 KOH contained the carbon dioxide from C-1 of glucose via the HMP. Set 2 KOH contained the carbon dioxide from the decarboxylation of pyruvic acid isolated from the medium in the main chamber of the reaction vessel.

From the ^{14}C data and glucose oxidation data it is apparent that all the glucose supplied was oxidized to pyruvate via E–D and H.M.P. pathways and none via E.M.P. pathway. It is of interest to note that in the first hour of oxidation, about 86% of the glucose was broken down to pyruvate by E–D pathway. Later, 65% of the glucose was utilized via E–D pathway and 35% via H.M.P. pathway.

The enzymes phosphoglyceromutase, enolase and pyruvic kinase catalyze respectively the formation of 2-phosphoglyceric acid from 3-phosphoglyceric acid, phosphoenolpyruvic acid from 2-phosphoglyceric acid and pyruvic acid from phosphoenolpyruvic acid. The presence of these three enzymes of the glycolytic scheme in *C. fumago* is shown by the formation of pyruvic acid from 3-phosphoglyceric acid in the presence of cell-free extracts of *C. fumago*. The results are given in Table VII.

TABLE VII

PYRUVIC ACID FORMATION FROM 3-PHOSPHOGLYCERIC ACID BY CELL-FREE EXTRACTS

The assay system contained 3-phosphoglyceric acid, 10 μmoles; 0.01 M Tris buffer (pH 7.0) 4 ml; ADP, 20 μmoles; sodium arsenite to give a final concentration of $5 \cdot 10^{-3}$ M; cell-free extract containing 14 mg protein; and distilled water to make a final volume of 12 ml. The cell-free extract was made by squeezing washed whole cells through a French pressure vessel at about 15000 lb/in² pressure. The supernatant fraction, after centrifuging this homogenate at 1200 × *g* for 20 min, was used as the cell-free extract. Pyruvic acid was estimated by the ceric sulfate method of KREBS AND JOHNSON[6].

Time (h)	*μmoles pyruvate formed endogenously*	*μmoles pyruvate formed when 3-PGA* was added*
0	0	0
2.5	0	0.8
5.0	0	1.0

* 3-PGA = 3-phosphoglycerate.

In order to determine why glucose was not phosphorylated by *C. fumago* before oxidation by glucose oxidase, cell free extracts were tested for the presence of glucokinase. The enzyme was assayed by the decrease in the acid-labile phosphate by the FISKE AND SUBBAROW procedure in an assay system containing ATP, Mg^{2+}, glucose, buffer and cell-free extracts. No loss of labile phosphates could be detected even at the end of 3 h of incubation at 30°. From this evidence and also from the fact that all of the glucose supplied was converted to gluconate and then to 2-ketogluconate by both whole cells and cell-free preparations, it is concluded the enzyme glucokinase is not present in *C. fumago*. Its absence could be one of the reasons for the lack of an E.M.P. pathway in *C. fumago*.

TABLE VIII

OXIDATION OF TRICARBOXYLIC ACID CYCLE INTERMEDIATES

The assay system contained washed cell suspension, 1.0 ml; substrate, 4 μmoles and phosphate buffer (pH 6.0) to make up the final volume of 3 ml.

Substrate added	*μl O_2 uptake in 4 h*
Glucose	279
Citric acid	201
α-Ketoglutaric acid	183
Succinic acid	204
Fumaric acid	201
l-Malic acid	190
Endogenous	67

The tricarboxylic acid cycle in C. fumago

The presence of an operative tricarboxylic acid cycle was demonstrated by showing that (a) several intermediates of the tricarboxylic acid cycle were utilized by the whole cells with concomitant O_2 uptake (Table VIII), (b) the sensitivity of the α-ketoglutarate utilization to the inhibitor arsenite at $3 \cdot 10^{-3}$ M concentration (Fig. 2), and (c) isocitric dehydrogenase was present in cell-free extracts (Table IX). However, the

activity of the isocitric dehydrogenase in the extract was very low. Efforts to demonstrate other dehydrogenases of the tricarboxylic acid cycle spectrophotometrically were unsuccessful. Apparently the various dehydrogenase enzymes of *C. fumago* were in some way inactivated during extraction by the various methods that were tried.

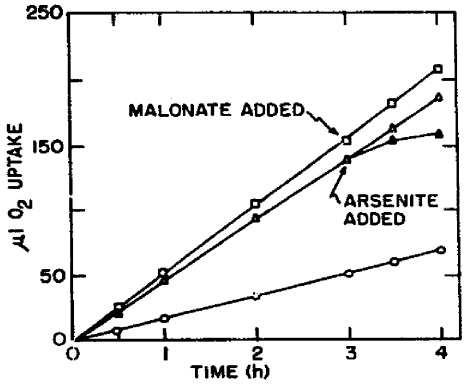

Fig. 2. Effect of inhibitors on α-ketoglutarate and succinate oxidation by whole cells. The experiment is described in Table VIII. In two sets of flasks containing α-ketoglutarate and succinate, 4 μmoles each, 0.2 ml each of 0.045 M sodium arsenite and 0.1 M of malonate respectively were tipped in from the side arm, 3 h after the start of the experiment. O—O, endogenous; △—△, α-ketoglutarate without added arsenite; ▲—▲, α-ketoglutarate with arsenite; □—□, succinate with or without added malonate.

TABLE IX

FORMATION OF α-KETOGLUTARATE FROM ISOCITRATE BY CELL-FREE EXTRACTS

The assay system in 12 ml contained: isocitric acid, 10 μmoles; $1.5 \cdot 10^{-3}$ M TPN, 0.5 ml; manganous chloride, 5 μmoles; sodium arsenite, $3 \cdot 10^{-3}$ M; 0.01 M Tris buffer (pH 7.0), 4 ml; cell-free extract, 2.0 ml; and distilled water, 3.5 ml. Incubation temperature was 30°. The cell-free extract containing 14 mg protein/ml was prepared as explained in Table VII. The α-ketoglutarate was estimated by the ceric sulfate decarboxylation method of KREBS AND JOHNSON[6].

Time (h)	*Endogenous α-ketoglutarate (μmoles)*	*α-Ketoglutarate formed with 10 μmoles of isocitrate (μmoles)*
0	0	0
2.5	0	1.3
5.0	0	2.3

DISCUSSION

Caldariomyces fumago evidently does not utilize the E.M.P. pathway for glucose dissimilation for, under anaerobic conditions, it produces neither carbon dioxide nor organic acids from glucose. The absence of glucokinase probably prevents this anaerobic activity. The lack of this enzyme also shows that *C. fumago* does not possess a typical H.M.P. pathway in which glucose is phosphorylated before oxidation. The initial oxidation of glucose therefore depends entirely on the molecular oxygen requiring glucose oxidase and gluconate oxidase. With these two enzymes the fungus converts all the glucose to 2-ketogluconic acid. Similar oxidations of nonphosphorylated glucose have been reported in both bacteria and fungi. In *Penicillium glaucum* and *Penicillium notatum* a flavoprotein-linked glucose oxidase has been reported. In Pseudomonads[13] a cytochrome-linked glucose oxidase has been reported. Among these organisms only the glucose oxidase from Penicillia is an auto-oxidizable flavoprotein with hydrogen peroxide as one of the end products. This Penicillial system is cyanide-and azide-insensitive. The glucose oxidase of *C. fumago* resembles that of

P. notatum in the following aspects: (a) 1.5 moles of oxygen consumed/mole of glucose converted to 2-ketogluconate, (b) hydrogen peroxide is one of the products of the enzymic action and (c) its insensitivity to 10^{-3} M cyanide and azide. From these data it is apparent that the primary oxidation of glucose in *C. fumago* is as follows:

$$\text{glucose} \xrightarrow[\nearrow\ O_2 \quad \nearrow\ H_2O \quad \searrow\ H_2O_2]{\text{glucose oxidase}} \text{gluconic acid} \xrightarrow[\nearrow\ \tfrac{1}{2}\,O_2 \quad \searrow\ H_2O]{\text{gluconic oxidase}} \text{2-ketogluconate}$$

The overall reaction is:

$$\text{glucose} + 1.5\ O_2 \longrightarrow \text{2-ketogluconate}$$

No information concerning the pathway by which fungi metabolize 2-ketogluconate is available. In *C. fumago* the metabolism of 2-ketogluconate probably involves the conversion of 2-ketogluconate to 6-phosphogluconate via the adaptive enzymes 2-ketogluconate kinase and 2-keto-6-phosphogluconic reductase. Such adaptive enzymes have been reported in certain bacterial systems[8–12]. In *C. fumago* it is evident that the enzyme gluconokinase was not present because, with gluconate as substrate, one half mole of oxygen per mole of gluconate was taken up and 2-ketogluconate but not 6-phosphogluconate was formed. The fact that mycelium from young shake cultures with low pH would use 2-ketogluconate only after a lag period, while the same culture would oxidise 6-phosphogluconate shows that in the normal metabolism of 2-ketogluconic acid, a period of adaptation was necessary prior to its utilization. This lag disappears when the cells are preincubated in 2-ketogluconate.

The pathway of 2-ketogluconate metabolism, found in *Acetobacter melanogenum*[14,15], in which the 2-ketogluconate is oxidized to 2,5-diketogluconate, then decarboxylated and reduced to the pentose arabinose, which in turn is converted to α-ketoglutaric acid, is not present in *C. fumago*, for pyruvate and not α-ketoglutarate is the product formed from 2-ketogluconate in the presence of the inhibitor arsenite.

The 6-phosphogluconate can then enter either the H.M.P. pathway or the E–D pathway. The fact that more than 1.6 μmoles of pyruvate were consistently produced from 1 μmole of glucose, 2-ketogluconate, or 6-phosphogluconate points to the E–D pathway as the major route for glucose utilization in *C. fumago* with the H.M.P. pathway playing a minor role.

The results of the experiments with [^{14}C]glucose conclusively show that both these pathways operate simultaneously in *C. fumago* and that approx. 65 % of the glucose is broken down by the E–D pathway and the rest by the H.M.P. pathway. The only known pathway through which the number one carbon of glucose can end up in the carboxyl carbon of pyruvate is the E–D pathway where the 6-phosphogluconate is dehydrated to give 2-keto-3-deoxy-6-phosphogluconate and this is cleaved with an aldolase to yield pyruvate from the first three carbons of glucose and glyceraldehyde 3-phosphate from the last three carbons of glucose. The following enzymes that are involved in pyruvate formation form glucose were individually demonstrated: glucose oxidase, gluconic oxidase, 3-phosphogluconic mutase, enolase and pyruvic kinase.

Because of the atypical pathways for the utilization of glucose, caused by the absence of glucokinase and gluconokinase, the terms modified Entner–Doudoroff

pathway (m. E–D pathway) and modified hexose monophosphate pathway (m. H.M.P. pathway) should be used to describe the pathways of glucose oxidation in *C. fumago*.

The presence of an operative tricarboxylic acid cycle in *C. fumago* was shown in a number of ways. There was consistent uptake of 6 moles or better of oxygen for every mole of glucose, 2-ketogluconate or 6-phosphogluconate utilized. The fungus utilized all the tricarboxylic acid cycle intermediates that were supplied as substrate. The presence of the enzyme isocitric dehydrogenase was demonstrated in a cell-free preparation of *C. fumago*.

The sensitivity to cyanide of about 80% of the oxygen uptake in the total oxidation of glucose and almost all the oxygen uptake in the oxidation of 2-ketogluconate and 6-phosphogluconate indicates the involvement of the cytochrome system operating in the coupled electron transport mechanism.

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